Quorum-sensing *Salmonella* selectively trigger protein expression within tumors

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Salmonella **that secrete anticancer proteins have the potential to eliminate tumors, but nonspecific expression causes damage to healthy tissue. We hypothesize that *Salmonella,* integrated with a density-dependent switch, would only express proteins in tightly packed colonies within tumors. To test this hypothesis, we cloned the *lux* quorum-sensing (QS) system and a GFP reporter into nonpathogenic *Salmonella*. Fluorescence and bacterial density were measured in culture and in a tumor-on-a-chip device to determine the critical density necessary to initiate expression. QS *Salmonella* were injected into 4T1 tumor-bearing mice to quantify GFP expression in vivo using immunofluorescence. At densities below 0.6×10^{10} cfu/g in tumors, less than 3% of QS *Salmonella* expressed GFP. Above densities of 4.2×10^{10} cfu/g, QS *Salmonella* had similar expression levels to constitutive controls. GFP expression by QS colonies was dependent upon the distance to neighboring bacteria. No colonies expressed GFP when the average distance to neighbors was greater than 155 μm. Calculations of autoinducer concentrations showed that expression was sigmoidally dependent on density and inversely dependent on average radial distance. Based on bacterial counts from excised tissue, the liver density (0.0079×10^{10} cfu/g) was less than the critical density (0.11×10^{10} cfu/g) necessary to initiate expression. QS *Salmonella* are a promising tool for cancer treatment that will target drugs to tumors while preventing damage to healthy tissue.**

bacterial anticancer therapy | quorum sensing | *Salmonella* | cancer | localized drug delivery

Bacteria that induce expression only in tumors have the potential to solve a critical problem with chemotherapy. Current cancer chemotherapeutic regimens have limited efficacy due to therapeutic resistance and systemic toxicity (1–3), which prevents the use of more aggressive dosage schemes (4). *Salmonella* are capable of overcoming these limitations because they preferentially accumulate in tumors, actively penetrate tumor tissue, and can be engineered to produce anticancer drugs in situ (5–11). *Salmonella* that only activate drug expression in tumors and not healthy tissue will reduce toxicity and allow for the use of more aggressive therapeutics. Constitutive, systemic expression of an anticancer drug would be toxic, due to low-level bacterial accumulation in healthy tissue (5). Because *Salmonella* accumulate almost 10,000-fold higher in tumors than other organs (5, 12), bacteria that sense density would provide a switch to distinguish between healthy and cancerous tissue.

Strict control over protein expression is essential for managing the timing and location of drug production. Precise triggering of expression can boost drug concentration within tumors while minimizing harmful side effects (6). *Salmonella* can be engineered to induce protein expression in response to molecular triggers, radiation, or hypoxia (6, 11, 13–18). Molecular triggers are limited because small molecules cannot diffuse deep into tissue (19–21). Radiation-inducible promoters are inherently leaky (11), which would lead to unwanted drug expression in healthy tissue. Promoters that respond to hypoxia would have difficulty treating micrometastases less than 2 mm that are typically well oxygenated (22).

Quorum-sensing (QS) bacteria can change their gene expression based on population density (23). The *lux* QS system induces expression of bioluminescent genes in marine bacterium *Vibrio fischeri*. The *lux* QS system consists of two genes: *luxI* and *luxR* (Fig. 1*A*). Autoinducer synthesis protein LuxI synthesizes the autoinducer *N*-3(oxohexanoyl)homoserine lactone (3OC6HSL). This autoinducer is specific to *V. fischeri* and cannot communicate with other species of bacteria (23). Transcriptional regulator protein LuxR activates in the presence of 3OC6HSL and induces transcription by binding to the promoter p(*luxI*) (24, 25). At low population density, low-level expression of LuxI synthesizes 3OC6HSL, which freely diffuses out of cells. As the population density increases, intracellular 3OC6HSL activates LuxR, creating a positive feedback loop which increases the production of any gene incorporated into the operon (25). The *lux* QS system has been used in previous research to trigger *Escherichia coli* invasion into cancer cells (26).

The spatial distribution of bacteria affects the activation of a QS switch (27). The concentration of a signaling molecule diminishes as it moves away from a cell, which decreases the likelihood of activating the QS switch (28). Clustering of bacterial cells prevents dilution of the signaling molecule and improves QS activation (29). These observations suggest that QS *Salmonella* would only activate when close to each other in tumor colonies (Fig. 1*B*).

To create a tumor-sensitive gene expression switch, we integrated the *lux* QS system and a fluorescence reporter into an attenuated *Salmonella* cancer vector. We hypothesized that QS Salmonella would (*i*) induce gene expression in response to high bacterial density, (*ii*) induce expression as the distance between

Significance

Nonpathogenic *Salmonella* localize to tumors and can be engineered to secrete anticancer proteins, but tumor-specific expression is essential to prevent systemic toxicity. To reduce unwanted side effects in healthy tissue, we integrated *Salmonella* with a quorum-sensing (QS) switch that only initiates drug expression in the tightly packed colonies present within tumors. Using an in vitro 3D-tumor-on-a-chip device and in vivo mouse models, we show that QS *Salmonella* specifically initiates protein expression within cancerous tissue while remaining uninduced in livers. Protein expression was triggered when inducer molecules from enough close neighbors reached a critical concentration. Because of these selective qualities, QS *Salmonella* are a promising tool for tumor-specific delivery of therapeutic proteins.

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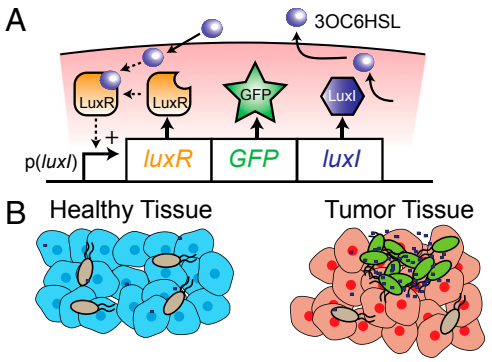

Fig. 1. QS bacterial drug delivery. (*A*) The p(*luxI*) promoter controls one operon consisting of genes encoding for proteins LuxR, GFP, and LuxI. LuxI produces the communication molecule 3OC6HSL. The p(luxI) promoter responds to LuxR protein bound to 3OC6HSL. As the density of bacteria increases, 3OC6HSL concentration increases within the cell, creating a positive feedback loop that increases transcription of the operon. (*B*) QS bacteria will only turn on expression in high-density colonies in tumor tissue. Gray and green bacteria represent uninduced and induced bacteria, respectively. Blue dots represent 3OC6HSL.

bacteria decreases, and (*iii*) only induce expression in tumor tissue. To test this hypothesis, fluorescence and density were measured and compared with constitutive controls to determine the basal level of QS expression. GFP expression was measured in bacterial cultures and an in vitro tumor-on-a-chip device to quantify the density required to trigger expression. QS and constitutive *Salmonella* were injected into tumor-bearing mice to quantify protein expression in vivo. Bacterial density was measured in tumors and livers. Immunofluorescence was used to quantify the spatial distribution of bacteria and GFP expression within tumors. A mathematical model was created to predict both the density and distribution of bacteria needed to induce protein expression in tissue. QS *Salmonella* will be an improvement over chemotherapy because it creates a sensitive switch that will only express protein therapeutics in tumors while remaining off in healthy tissue.

Results

Density Dependence of GFP Expression in Vitro. QS *Salmonella* induced GFP expression both in flasks and in vitro tumor tissue only at high density (Fig. 2). At densities less than 0.5×10^8 cfu/mL, QS *Salmonella* did not express GFP (Fig. 2*A*). Above the critical density of 10^8 cfu/mL, QS *Salmonella* expressed significant amounts of GFP (Fig. 2*A*; $P < 0.05$). Constitutive controls expressed GFP, regardless of density ($P < 0.05$). Constitutive *Salmonella* were used as controls because GFP expression was not dependent on an external inducer in these bacteria. GFP expression in constitutive controls was detected at densities as low as 0.25×10^8 cfu/mL (Fig. 2*A*). The critical density of GFP expression was robust and did not change with culture history (Fig. 2*B*). For cultures grown to different densities before dilution (0.5×10^8 cfu/mL and 5×10^8 cfu/mL), GFP expression was consistently induced at 10^8 cfu/mL (Fig. 2*B*). Cultures grown to higher density before dilution, however, had greater GFP expression with time (Fig. 2*B*; $P < 0.05$) because of residual LuxI and LuxR molecules in the bacteria (30).

In tissue in a microfluidic device (31), QS *Salmonella* only expressed GFP in high-density colonies (Fig. 2 *C* and *D*). Bacterial accumulation began 10 h after inoculation. By 53 h, tumor tissue containing constitutive bacterial controls expressed GFP throughout the entire tissue (Fig. 2*C*). Bacteria of both strains colonized the entire tissue. 38 h after bacterial injection, tumor tissue accumulated with QS *Salmonella* had pockets of GFP expression within distinct colonies. Tissue with sparse colonization contained no GFP expression (Fig. 2*C*). The area of tissue with GFP expressing bacteria was greater in constitutive controls (97%) than QS *Salmonella* (45%, Fig. 2*D*; $P < 0.05$).

***Salmonella* Distribution in Tumor-Bearing Mice.** QS *Salmonella* and constitutive controls preferentially accumulated in tumor tissue compared with healthy tissue (Fig. 3). Bacterial density, based on plating of minced tissue, was 89-fold and 387-fold greater in tumor tissue than liver tissue for QS and constitutive *Salmonella*, respectively (Fig. 3*A*; $P < 0.05$). There was no statistical difference between the QS ($n = 5$ mice) and constitutive ($n = 5$) bacterial densities in tumors or livers (Fig. 3*A*; $P > 0.3$). GFP was present in all tumors. Expressing colonies are difficult to see in these macroscopic images because of their small size (Fig. S1). Tumor tissue removed at 9 and 24 d after bacterial injection both contained GFP, indicating persistent gene expression over this time range (Fig. 3*B*). Because of the low density, no *Salmonella* were observed in liver sections by immunofluorescence (Fig. 3*C*). In tumors, most colonies formed in regions of low bacterial density. Local density was defined as the number of *Salmonella* within a 197 μm (r_c; 150 pixel) radius around a colony. Colonies were groups of contiguous bacteria distinctly separate from neighbors (Fig. 3*D*, *Inset*). Eighty-three percent of QS ($n = 84{,}213$ colonies) and constitutive ($n = 133{,}305$) *Salmonella* colonies were at a density of 0.3×10^{10} cfu/g or less (Fig. 3*D*). Of the QS *Salmonella*, 0.7% were at densities higher than 3.3×10^{10} cfu/g. The highest density of a QS *Salmonella* colony was 5.23×10^{10} cfu/g (Fig. 3*D*).

Density-Controlled Protein Expression in QS Colonies in Tumors. Protein expression by QS *Salmonella* was dependent on local density

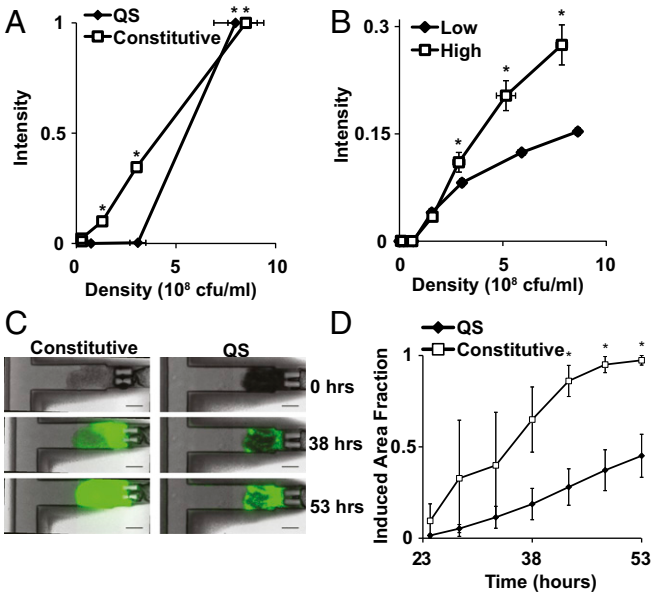

Fig. 2. In vitro behavior of QS *Salmonella*. (*A*) QS *Salmonella* only expressed GFP at densities above 1×10^8 cfu/mL (*$P < 0.05$). Constitutive controls expressed GFP at all densities. (*B*) Before measurement, QS cultures were grown to either 0.5×10^8 cfu/mL (low density) or 5×10^8 cfu/mL (high density and induced) and then diluted to 0.001×10^8 cfu/mL. High dilution density cultures had greater expression (*$P < 0.05$). Fluorescence was normalized to constitutive controls at 6×10^8 cfu/mL. (*C*) 38 h after injection into tumor tissue in a microfluidic tumor-on-a-chip device, QS *Salmonella* expressed GFP only within distinct bacterial colonies. Constitutive *Salmonella* expressed GFP throughout the tissue regardless of bacterial concentration. Bacteria took 10 h to colonize tissue and were not present at 0 h. (Scale bar, 100 μm.) (*D*) Area fraction of tissue with GFP expression was less for tissue treated with QS *Salmonella* compared with constitutive controls after 40 h (*$P < 0.05$).

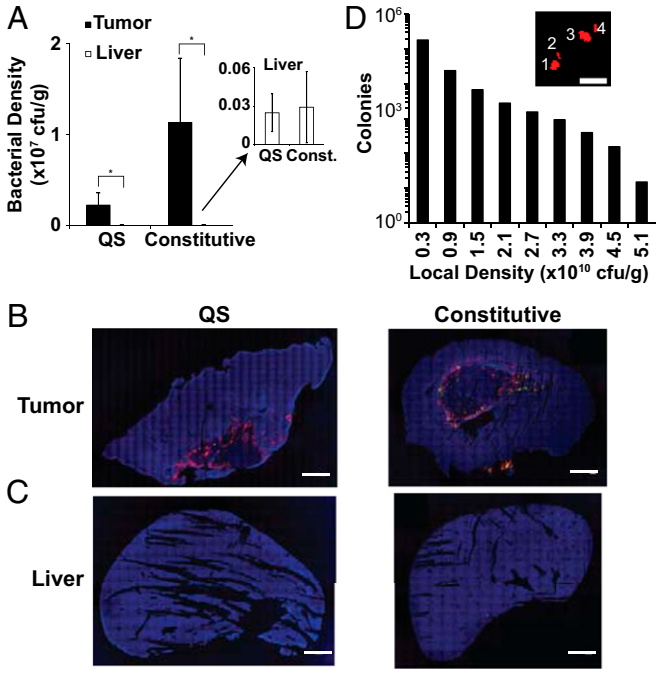

Fig. 3. *Salmonella* distribution within tumor-bearing mice. (*A*) Bacterial density was higher in tumors (solid bars) than livers (open bars) for mice administered both QS and constitutively expressing *Salmonella* (*$P < 0.05$). (*Inset*) Liver densities were small compared with tumor densities, but bacteria were present in most livers. (*B*) *Salmonella* (red) and GFP (green) distribution in 4T1 tumors injected with QS *Salmonella* or constitutive controls. Sections were counterstained with DAPI (blue). (*C*) No *Salmonella* or GFP were observed in any liver section using immunofluoresence. (Scale bar, 5 mm.) (*D*) Most *Salmonella* colonies were located in low-density regions. Local density is the number of bacteria within a 197-μm circle surrounding each colony. Colonies were groups of contiguous bacteria distinctly separate from neighbors (*Inset*). (Scale bar, 25 μm.)

(Fig. 4). In high-density regions, QS colonies expressed GFP (Fig. 4 *A*, *i*). Local density was high when colonies were large (as in Fig. 4 *A*, *i*) or were surrounded by many close neighbors. In low-density regions, small QS colonies did not produce GFP (Fig. 4 *A*, *ii*). In comparison, constitutive colonies expressed GFP regardless of size or local density (Fig. 4 *A*, *iii* and *iv*). Both large (Fig. 4 *A*, *iii*) and small (Fig. 4 *A*, *iv*) constitutive colonies expressed GFP.

The relationship between the fraction of GFP-expressing QS colonies and local density was sigmoidal (Fig. 4*B*). In comparison, the relationship for constitutive controls was linear and constant across all densities. At low densities, the fraction of induced QS colonies was low. Below a density of 0.6×10^{10} cfu/g, the induced fraction was 10-fold lower than controls (Fig. 4*B*; $P < 0.05$). At higher densities, the induced fraction was close to the maximum value of 1. Above 4.8×10^{10} cfu/g, 93% of QS colonies were induced. The difference between colonies at low ($<0.6 \times 10^{10}$ cfu/g) and high ($>4.8 \times 10^{10}$ cfu/g) density was 21-fold ($P < 0.05$). The fractions of expressing QS colonies at densities less than 4.2×10^{10} cfu/g were all less than the fraction of expressing control colonies at the lowest density of 0.3×10^{10} cfu/g ($P < 0.05$). Below a threshold density of 3.0×10^{10} cfu/g, the fraction of induced QS colonies was seven times less than constitutive controls (Fig. 4*C*; $P < 0.05$). Above this threshold, the fraction of induced QS colonies increased sixfold ($P < 0.05$) and was equivalent to constitutive controls (Fig. 4*B*; $P < 0.05$).

Proximity Between Colonies Controlled Expression. The percentage of QS colonies expressing GFP was greater for colonies closely surrounded by neighbors (Fig. 5). The key descriptor of spatial distribution was average radial distance, which was defined as the location-weighted average of distances between a colony and all neighboring bacteria within 197 μm (Fig. 5*A*). Colonies with equal densities but different average radii had different GFP-expression patterns (Fig. 5*B*). A colony with close neighbors, at an average radial distance of 74 μm, expressed GFP (Fig. 5 *B*, *i*). In comparison, a colony at the same density, but with distant neighbors (at a radius of 145 μm, or 71 μm farther away) was not induced (Fig. 5 *B*, *ii*). The average radius to neighboring bacteria affected GFP expression (Fig. 5*C*) in colonies in regions with density greater than 0.11×10^{10} cfu/g. In this range, the percentage of colonies expressing GFP was linearly and inversely dependent on average radius. At low densities, below 0.11×10^{10} cfu/g, induction was sparse (Fig. 4*B*) and not correlated with radius. The average expression fraction for all moderate and high-density colonies was 0.09 (Fig. 5*C*). The fractions of induced colonies with close ($3 < r < 58$ μm) and distant ($87 < r < 166$ μm) neighbors were significantly greater ($P < 0.05$) and less ($P < 0.05$) than the average, respectively. The lowest and highest radii measured were 3 and 166 μm. No colonies with neighbors farther away than 155 μm expressed GFP.

Production and Diffusion of 3OC6HSL in Tumor Tissue. The density and spatial distribution of bacteria in tumors predicted protein expression by individual colonies (Fig. 6). These two dependencies showed how QS controlled expression. At both higher density and shorter average distance between bacteria, the fraction of induced colonies was greater (Fig. 6*A*). For QS bacteria, protein expression was induced by 3OC6HSL. In tumors, two mechanisms controlled the concentration of 3OC6HSL: production by surrounding bacteria and diffusion through interstitial tissue (Fig. 6*B*). A target colony surrounded by few distant colonies (Fig. 6 *B*, *i*) would have had a low local 3OC6HSL concentration (Fig. 6*B*). A colony with twice the number of source colonies (Fig.

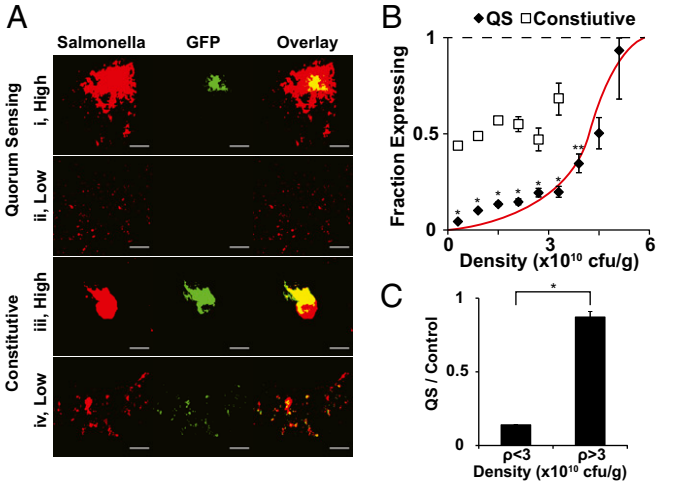

Fig. 4. Density dependence of GFP expression by QS *Salmonella*. (*A*) Colonies of QS and constitutive *Salmonella* (red) in 4T1 tumors and associated GFP (green) for areas of low and high density. Yellow indicates areas of colocalization between *Salmonella* and GFP. (Scale bar, 100 μm.) (*B*) GFP expression dependence on *Salmonella* density. The relationship between expression and density was linear for constitutive controls ($n = 133{,}305$ colonies) and sigmoidal (red line) for QS *Salmonella* ($n = 84{,}213$ colonies). At all densities less than 4.2×10^{10} cfu/g, the fraction of GFP-expressing QS colonies was less than the constitutive control colonies at the same density (*$P < 0.05$). Below 4.2×10^{10} cfu/g, the expressing fraction of QS colonies was less than control colonies at the lowest density, 0.3×10^{10} cfu/g (* and **$P < 0.05$). (*C*) The ratio of QS to control expressing fractions was significantly greater at densities above 3.0×10^{10} cfu/g compared with the densities below this threshold (*$P < 0.05$). Above 3.0×10^{10} cfu/g the QS:control ratio was close to 1.

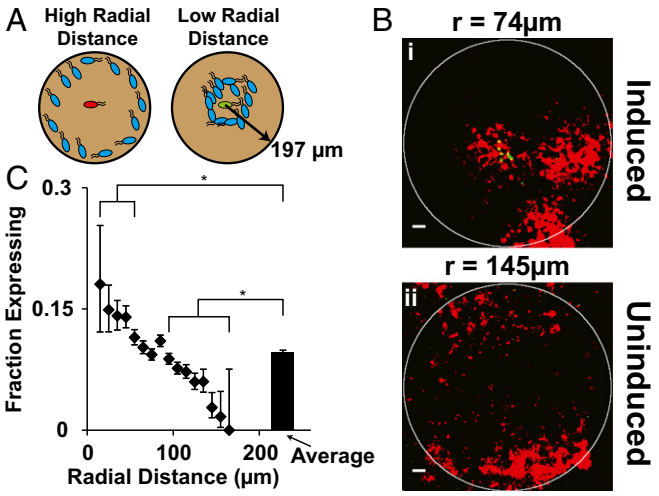

Fig. 5. Dependence on spatial distribution. (*A*) A QS *Salmonella* colony (*Center*) was more likely to express GFP if the average radial distance to its neighbors was shorter. Red, green, and blue bacteria represent uninduced, induced, and neighboring colonies, respectively. Density and radial distance was measured within circles of radius 197 μm (150 pixels) around colonies. (*B*) GFP expression was different for two colonies at the same density (1.2×10^{10} cfu/g) with different spatial distributions. A colony with an average radial distance to its neighbors of 74 μm expressed GFP (*B*, *i*), but a colony with an average radial distance of 145 μm did not (*B*, *ii*). (Scale bars, 100 μm.) (*C*) The fraction of colonies expressing GFP was greater for colonies with close compared with far neighbors, and with densities greater than 0.11×10^{10} cfu/g (n = 50,145 colonies). The expressing fraction was greater than the average for colonies with average radial distances less than 58 μm (*$P < 0.05$) and less than the average for distances greater than 87 μm (*$P < 0.05$). At an average distance of 155 μm, the fraction expressing was zero.

6 *B*, *ii*) would have had double the 3OC6HSL concentration. Similarly, a colony that was closer to source colonies (Fig. 6 *B*, *iii*) would have had a higher 3OC6HSL concentration.

To quantify these mechanisms, the 3OC6HSL concentration around source and target colonies was modeled as a coupled production–diffusion system.

$$\frac{\partial C_s}{\partial t} = \frac{D_{eff}}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial C_s}{\partial r}\right) \qquad m = \frac{m_{\max}}{2}\left[1+\tanh\left(\frac{\overline{\rho}-\overline{\rho}_{crit}}{\sigma}\right)\right]. \quad [1]$$

Around a source colony, the 3OC6HSL concentration (C_s) was dependent on the production rate (m) and an effective diffusion coefficient (D_{eff}) in heterogeneous tumor tissue (*SI Materials and Methods*). Above a critical density ($\overline{\rho} \gg \overline{\rho}_{crit}$), 3OC6HSL was produced at the maximum rate (m_{max}). Production decreased at low density with sensitivity (σ). At steady state (see *SI Materials and Methods* for derivation), the normalized source-colony concentration ($\overline{C}_s = C_s/C_q$) was inversely related to normalized radial distance ($\overline{r} = r/r_c$) by dimensionless production–diffusion (Q).

$$\overline{C}_s = \frac{Q}{\overline{r}} \qquad Q = \frac{m}{4\pi D_{eff} C_q r_c} \qquad \overline{C}_t = \overline{\rho}\overline{C}_s = Q\frac{\overline{\rho}}{\overline{r}}. \quad [2]$$

The reference concentration (C_q) was the concentration at which 50% of QS colonies were induced. The concentration at each target colony ($\overline{C}_t$) was equal to the contribution of 3OC6HSL from the total number of source colonies ($\overline{\rho}$) within radius r_c (Fig. 6 *B*, *ii*). Each target colony was at an average radial distance ($\overline{r}$) from all surrounding source colonies. The probability that a target colony was induced (α) was dependent on the 3OC6HSL concentration ($\overline{C}_t$) and the minimum probability (β; Fig. 6*C*).

$$\alpha = \frac{1}{1+e^{\beta(\overline{C}_t-1)}}. \quad [3]$$

At increasing 3OC6HSL concentrations, the probability of GFP expression (α) and the fraction of induced colonies both increased (Fig. 6*C*). Dimensionless production–diffusion, Q, was 1.34 ($P < 1 \times 10^{-15}$), indicating that the system was moderately diffusion limited (Table 1). The normalized critical induction concentration ($\overline{C}_{crit}$) was 0.38 (Fig. 6*C*). Based on previously measured values of C_{crit} (30, 32) and D_{eff} (12, 32–34), m_{max} was 53,000 molecules·s^{-1} per bacterium.

The predicted fraction of induced colonies was greater at high density and low radius (Fig. 6*D*). This dependence was caused by the proportional and inverse relationships of 3OC6HSL concentration to $\overline{\rho}$ and $\overline{r}$, respectively (Eq. **2**). At small radii, the predicted fraction of induced colonies was close to 1, regardless of density (Fig. 6*E*). Similarly, at low density, the predicted fraction of induced colonies was close to zero, regardless of radius (Fig. 6*F*). At high average radii between colonies, a greater density was required to produce a $\overline{C}_t$ greater than $\overline{C}_{crit}$ and induce expression (Fig. 6*G*). Inversely, at low radii, 3OC6HSL concentration was less dependent on density (Fig. 6*H*). Below the critical density ($\rho_{crit} = 0.11 \times 10^{10}$ cfu/g), protein production (m, Eq. **1**) and the 3OC6HSL concentration were both zero (Fig. 6*I*). The dependence of production (m) on density was almost binary because the sensitivity ($\sigma = 6.84 \times 10^3$ cfu/g) was nearly six orders of magnitude smaller than ρ_{crit} (Table 1).

Based on the number of bacteria within the liver (1.51×10^4 cfu/g; Fig. 3*A*), ~47 bacteria were located within each liver section. The absence of visible colonies in the immunofluorescent liver images (Fig. 3*D*) indicates that bacteria were sparsely distributed as individuals within the tissue. In the extreme case that these bacteria were all located within a single colony, the density would be 7.92×10^7 cfu/g. Because this density (ρ_{liv}) was less than ρ_{crit} (Fig. 6*I*), 3OC6HSL production was independent of radius and spatial distribution (Fig. 6*I*). At this maximum possible liver density, production, 3OC6HSL concentration, and protein expression would have all been zero.

Discussion

Administering *Salmonella* with the ability to change gene expression in a density-dependent manner will initiate protein expression within tumors and has the potential to reduce systemic toxicity. We have shown that *Salmonella* integrated with a QS trigger turn on protein expression in tightly packed high-density colonies within tumors, while remaining off in low-density colonies. A mathematical model of 3OC6HSL concentration in tumor tissue was used to determine the mechanisms of QS protein expression. The model predicted that QS *Salmonella* will not trigger protein expression in healthy tissue. When *Salmonella* were administered with a constitutive trigger, protein expression was observed in low-density colonies and in individual *Salmonella* with no surrounding neighbors. A bacterial cancer therapy with a QS triggering system will prevent therapeutic protein release in healthy tissue and maximize therapeutic effect in tumors.

The density of QS *Salmonella* in livers and the critical density needed to trigger the QS system render the possibility of gene expression unlikely in healthy tissue. Mathematical modeling predicts that QS *Salmonella* would remain off (Fig. 6*I*) at the density measured in liver tissue (Fig. 3*A*). Constitutive controls, on the other hand, expressed GFP at the lowest possible detectable density in tumor tissue (Fig. 4*B*), indicating that the constitutive *Salmonella* would express GFP everywhere, including the liver. Constitutive expression of toxic proteins in livers or other healthy organs, even at low rates, could have detrimental effects on the host. QS *Salmonella* can overcome these therapeutic limitations by specifically triggering drug expression within tumors without

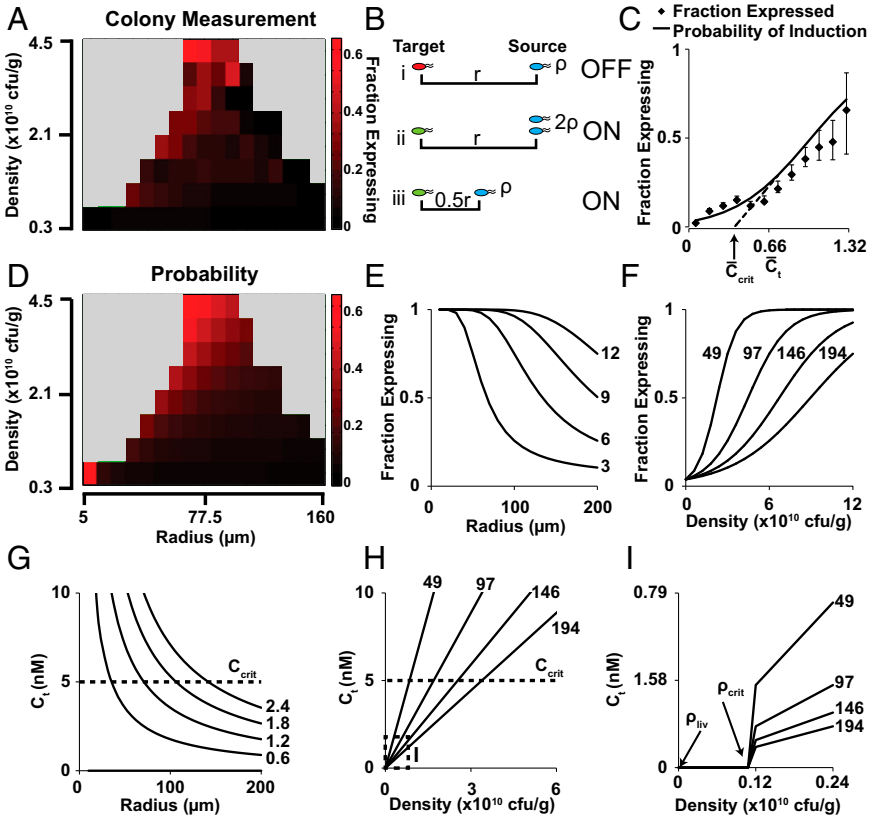

Fig. 6. Calculated 3OC6HSL concentration predicts GFP expression. (*A*) The fraction of colonies (n = 84,213) that expressed GFP was greater (more red) at high densities (*Top*) and short radial distances (*Left*). (*B*) Compared with an uninduced colony (*B*, *i*, red), a target colony was more likely to express GFP (green) if (*B*, *ii*) it was surrounded by more source colonies (blue) or (*B*, *iii*) the distance to source colonies was shorter. (*C*) The fraction of expressing colonies and the predicted probability of induction (α), were lower for colonies with lower calculated concentrations of 3OC6HSL ($P < 1 \times 10^{-15}$). The critical 3OC6HSL concentration (C_{crit}) was $\bar{C}_t = 0.38$. (*D*) The predicted fraction of expressing colonies, α, was higher at high density and low average radial distance, matching colony measurements in tumor sections (*A*). (*E* and *F*) Predicted α-fractions were lower at longer average radial distances (*E*) and higher at higher densities (*F*). Numbers to the right are density ($\times 10^{10}$ cfu/g) for *E* and *G*, and radius (μm) for *F*, *H*, and *I*. (*G* and *H*) The predicted 3OC6HSL concentration at target colonies ($\bar{C}_t$) was lower at higher average radial distances (*G*), and higher at higher densities (*H*). (*I*) Expanded range of *H*. At low densities below $\rho_{crit} = 0.11 \times 10^{10}$ cfu/g, the predicted 3OC6HSL concentration was zero. At the maximum possible bacterial density in liver ($\rho_{liv} = 0.00792 \times 10^{10}$ cfu/g), no 3OC6HSL was produced, regardless of spatial distribution.

causing unintended side effects. It is also unlikely that *Salmonella* would grow in off-target tissues to densities that would induce expression. Experiments with cynomolgus monkeys have shown that, after initial accumulation following injection, *Salmonella* are eliminated from most organs by 30 d (35).

QS *Salmonella* have important advantages over other proposed mechanisms of bacterial drug delivery. Integrating *Salmonella* with a robust QS triggering system enables the use of aggressive therapeutic proteins, such as *Staphylococcus aureus* α-hemolysin (SAH). SAH kills cells quickly and is effective against therapeutically resistant tissue (36, 37). Systemic delivery of ubiquitously toxic molecules is not a viable treatment strategy because all tissues would be damaged. When controlled by the QS system, SAH is only released from high-density colonies, where it kills cancer cells and tumor tissue (Fig. S2). At bacterial densities considerably higher than the density in livers, SAH is not produced and no toxicity is observed (Fig. S2).

With QS, no external inducer is needed to initiate expression after colonization. Previous strategies with external inducers have been problematic. Inducers must overcome both clearance from the body and diffusion barriers into tissue. Without the need for an external inducer, a QS system is not reliant on the delivery of a small molecule to maintain therapeutic expression levels. Persistent gene expression was observed in tumor tissue as late as 24 d after injection (Fig. 3*C*). From a clinical standpoint, this enables continual drug production and an increased therapeutic effect over a longer period.

Table 1. Autoinducer transport parameters

Name	Parameter	Value
Dimensionless production–diffusion	Q	1.34
Critical density	ρ_{crit}	0.11×10^{10} cfu/g
Density sensitivity	σ	6.84×10^{3} cfu/g
Minimum probability	β	−3.2

The sensitivity of this density-dependent switch suggests that QS *Salmonella* will turn on in undetected metastatic legions. The QS system turns on at a critical density of 0.11×10^{10} cfu/g in tumor tissue (Fig. 6*H*). In previous work, *Salmonella* were shown to accumulate in liver metastases, at a density of 5.28×10^{10} cfu/g (7). Assuming a uniform distribution, all colonies at this density would have 3OC6HSL concentrations over C_{crit} (Fig. 6*G*). In comparison, *Salmonella* were at a density of 0.06×10^{10} cfu/g in the surrounding hepatic parenchyma (7), almost half the density required for the QS system to activate (Fig. 6*H*).

Increasing the number of colonies within tumor tissue has the potential to increase drug production, due to the important roles diffusion and bacterial spatial distribution play in triggering the QS system. QS *Salmonella* turn on protein expression at densities of 10^8 cfu/mL in flasks (Fig. 2*A*), but mixing ensures that 3OC6HSL is well distributed and not affected by diffusion. In tumor tissue, however, the QS switch turned on at densities of 11×10^8 cfu/mL, almost 10-fold higher than in flasks (Fig. 6*H*), assuming a tissue density of 1 g/mL. The increase in bacterial density was caused by the distance necessary for 3OC6HSL to diffuse through tissue once it was produced. Below this critical density, there were not enough individuals producing 3OC6HSL to induce expression, no matter how tightly packed they were (Fig. 6*I*). In addition, 3OC6HSL must overcome loss through hyperpermeable blood vessels, dissipation by lymphatic flows, and the heterogeneous environment of tumor tissue.

Administration of QS *Salmonella* with exogenous lipid A (12) and enhanced motility (12, 38) would increase bacterial density and improve distribution within tumors. Combined, these effects could increase density 253-fold and increase overall drug production. Mathematical modeling predicted that as the density of bacteria increased, bacteria did not have to be as tightly packed together to induce the QS switch (Fig. 6*G*). Therefore, coupling enhanced-motility QS *Salmonella* with lipid A administration could have an exponential effect on drug production.

Conclusion

Salmonella integrated with a QS triggering system creates a drug delivery vehicle that improves upon existing therapies. QS *Salmonella* only initiate protein production within tumors and not in healthy tissue. These bacteria maintain continuous therapeutic production due to persistent expression. No external inducer is required to initiate drug production. The QS switch is not dependent on cell surface markers that are unique to specific tumor types. Because of these targeting abilities, QS *Salmonella* are a promising tool to deliver therapeutic proteins and treat cancerous tissue and metastases.

Materials and Methods

Detailed methods are found in *SI Materials and Methods*.

In Vitro Bacterial Density and GFP Expression Analysis. A robust density switch was created in *Salmonella* by transforming a QS architecture, in which all genes are under control of the *p*(*luxI*) bidirectional promoter (Fig. S3), into VNP200010 (*msbB*$^-$, *purI*$^-$, *xyl*$^-$, *asd*$^-$) a nonpathogenic *Salmonella* strain (Fig. 1*A*). To measure density dependence of GFP expression, *Salmonella* were grown from single colonies in flasks and optical density and fluorescence were measured hourly. A microfluidic tumor-on-a-chip device containing LS174T colon carcinoma cells was used to measure bacterial protein expression in tissue. *Salmonella* were administered to devices for 1 h and then switched to bacteria-free medium. Transmitted and fluorescence images were acquired for 30 h, starting 23 h after bacterial inoculation (Olympus), and were analyzed using ImageJ (NIH Research Services Branch).

In Vivo *Salmonella* Administration and Analysis. QS and control *Salmonella* were injected via the tail vein into mice with 500-mm^3 s.c. 4T1 mammary tumors. Mice were killed when tumors reached 2,000 mm^3. Excised tumors and livers were cut in half. One half was plated on LB-agar plates and colonies were counted after 24 h. The other half was embedded in paraffin, sectioned, and probed with antibodies against GFP and *Salmonella*. Fluorescence images were thresholded and colonies were identified. Local density was determined by counting the number of bacteria in a 150-pixel (197-μm) circle around each colony. Average radial distances of neighboring bacteria were determined by counting *Salmonella* within 5-pixel-wide annuli and weighting by the annulus area. Colonies were considered to be induced if a GFP pixel was within 25 pixels. All animal procedures were approved by Baystate Medical Center Institutional Animal Care and Use Committee, and were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (39).

Mathematical Modeling of 3OC6HSL Diffusion. A mathematical model was used to predict the concentration of 3OC6HSL in tumor tissue, which has an analytical solution (*SI Materials and Methods*). Parameters ρ_{crit}, σ, β, and Q were determined by binomial regression of the logistic probability function (Eq. **3**) and the predicted 3OC6HSL concentrations (Eq. **2**) for each colony. The value of C_{crit} was determined by linearly extrapolating from the concentration of maximum slope in Eq. **3** to the concentration at which $\alpha = 0$.

Statistical Analysis. For results obtained in bacterial cultures, microfluidic devices, and tissue plating experiments, errors are reported as SEMs. Hypotheses were tested using Student's *t* test with a significance level of $P < 0.05$. For results obtained by colony analysis in tumor sections, errors are reported as 95% Clopper–Pearson binomial confidence intervals, with individual colonies as biological replicates. Hypotheses were tested using Fisher's exact test with a significance level of $P < 0.05$.

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